# SEROLOGICAL STUDIES OF THE ROOT-NODULE BACTERIA.

## IV. FURTHER ANALYSIS OF ISOLATES FROM TRIFOLIUM AND MEDICAGO.

By Hilary F. Purchase, J. M. Vincent and Lawrie M. Ward,
School of Agriculture, University of Sydney.

[Read 28th March, 1951.]

*Synopsis.*

The present paper summarizes serological data accumulated over a period of about ten years in respect of reference strains of *Rhizobium trifolii* and *Rh. meliloti*. Over this time the antigenic properties of the organisms have shown a high degree of stability and the technique has proved useful for the typing of isolates in field and laboratory studies.

To obtain adequate typing it is necessary to distinguish flagellar and somatic reactions. The latter on its own permits more strains to be distinguished than the former, but maximum differentiation requires both to be taken into account.

Extending the method of analysis used in the earlier papers it has been found that the description of the 12 isolates of *Rh. trifolii* requires at least 2 flagellar and 9 somatic antigens. The corresponding figures for the 16 isolates of *Rh. meliloti* are 4 and 15.

## Introduction.

Since the application of improved agglutination techniques to the study of root-nodule bacteria (Vincent, 1941, 1942) fair use has been made of these criteria for the classification and identification of serological strains in field and laboratory studies (Hughes and Vincent, 1942; Kleczkowski and Thornton, 1944; Vincent, 1944 and 1945; Purchase and Vincent, 1949; Read, 1950, private communication). Whilst there is no apparent relationship between serological constitution and the organism's behaviour in association with the host, the method remains a useful technique for distinguishing and grouping strains, a means of studying field distribution, and for "labelling" material for laboratory and field studies.

A comparison of our recent results with those reported in the earlier papers has shown a high degree of stability in antigenic properties; few cases have occurred where a culture has shown any significant change in this regard. The antigenic constitution appears in fact more stable than other characteristics (*cf.*, for example, Kleczkowski and Thornton, 1944; Nutman, 1946).

The earlier papers from this laboratory included reasonably detailed analyses of several strains of both species. The number of fully studied strains has been added to in the intervening years so that a fair battery of testing sera is now available. It has been thought worthwhile, therefore, to record these further studies which provide the basis of our more recent investigations.

## Experimental.

*Organisms used for the development of antisera.*—The isolates are identified by collection numbers and are mostly described in the earlier papers: *Rh. meliloti* (Vincent, 1941) and *Rh. trifolii* (Vincent, 1942). No. 204 has been added to the clover strains, having been obtained as "Clover F" strain from Dr. H. G. Thornton, Rothamsted Experiment Station, England. *Rh. meliloti* No. 52 originated from *Medicago hispida* var. *denticulata* growing at Warracknabeal, Victoria. Additionally it is worth noting that strains 7, 8, 10 and 12 came to us from the United States, the first three from the collection of the University of Wisconsin, and the last via the N.S.W. State Department of Agriculture.

*Methods.*—The methods of obtaining cultures and using them as antigens for the development and testing of antisera have been largely maintained as in the first paper (Vincent, 1941). It has been generally advantageous to obtain an earlier reading for flagellar (*H*) agglutination at about one hour and advisable to check somatic (*O*) agglutination with heated antigen, particularly in the presence of flagellar agglutina-

tion. Occasional difficulties have been encountered with a less voluminous flagellar agglutination but, provided a two-day motile culture of sufficient density is used, the result has been almost always satisfactory. We have not found it necessary to distinguish *H* by the use of *O*-absorbed sera or by the removal of *O* antibody by heat, although some workers might well prefer this added check (Kleczkowski and Thornton, 1944).

## Results.

*Strains of Rh. trifolii.*—Tables 1 and 2 give the results for flagellar and somatic reactions respectively.

Two major groupings of flagellar antigens are revealed, one represented by strain 36, and the other by strain 46. Following the 1942 treatment these have been classed A and B respectively. The earlier paper also showed by absorption tests that the five members of the first group tested on that occasion were identical. Flagellar reactivity has now been found between 157, 161 and 46—a result at variance with the earlier findings but supported by the agreement between reciprocal tests.

As has been found generally in these species, somatic antigens provide more groupings than do the flagellar. On the basis of what we now know of the somatic cross reactions of these strains the minimum number of somatic antigens would have to be extended from three to seven. Absorption tests have been applied in some detail to the 94–160 group of Table 2, and make it necessary to bring the number of antigens to nine. Some irregularities seem to be associated with antigen II of 94, 61, 111 and 161

Table 1.

*Flagellar Cross Reactions of* Rhizobium trifolii.

| Antigens. | Sera. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 36 | 108 | 61 | 111 | 94 | 91 | 160 | 204 | 46 | 157 | 161 | 64 |
| 36 | *3* 3 | 3 3 | 2 3 | 2 2 | 2 | 2 3 | 2 | 2 | –– | – | – | – |
| 108 | 3 3 | *3* 3 | 3 3 | 2 2 | 2 | 3 3 | 2 | 2 | –– | – | – | – |
| 61 | 3 3 | 3 3 | *3* 3 | 2 2 | 2 | 2 3 | 2 | 2 | –– | – | – | – |
| 111 | 2 3 | 3 3 | 3 3 | *2* 2 | 2 | 2 2 | 2 | 2 | –– | – | – | – |
| 94 | 3 3 | 2 | 3 | 2 | *3* | 2 | 2 | 2 | –– | – | – | – |
| 91 | 3 3 | 3 3 | 3 3 | 2 2 | 2 | *2* 2 | 2 | 2 | –– | – | – | – |
| 160 | 3 3 | 2 | 2 | 1 | 2 | 2 | *3* | 2 | –– | – | – | – |
| 204 | 3 | 2 | 2 | 2 | 2 | 2 | 1 | *3* | – | – | – | – |
| 46 | –– | –– | –– | –– | – | –– | – | – | *3* 3 | 2 | 2 | 3 |
| 157 | –– | – | – | – | – | – | – | – | 2 – | *3* | 2 | 0–1 |
| 161 | –– | – | – | – | – | – | – | – | 2 – | 2 | *3* | 0–1 |
| 64 | –– | – | – | – | – | – | – | – | 3 3 | 2 | 2 | *3* |

*Note.*—Numbers indicate highest dilutions showing agglutination, viz. 1 = 1/25 to 1/50, 2 = 1/100 to 1/200, 3 = 1/400 to 1/800, 4 = 1/1600 to 1/3200 or greater.
Top right-hand figure shows earlier result.

and indicate the possibility that the antigen might be composite and its components variable in their relative proportions between strains and in the one strain at different times.

The findings can now be summarized:

| Isolate. | Minimal Antigenic Composition. Flagellar. | Somatic. |
|---|---|---|
| 36, 108 | A | I |
| 94 | A | II, IV, VII |
| 61, 111 | A | II, V |
| 160 | A | IV, VII |
| 91 | A | III |
| 204 | A | IX |
| 64 | B | I |
| 161 | B | II, IV, VI |
| 46 | B | III |
| 157 | B | VIII |

Sera of the six strains underlined have been used for typing field isolates (Purchase and Vincent, 1949) and include representatives of both flagellar types and eight of the somatic antibodies.

TABLE 2.

*Somatic Cross Reactions of* Rhizobium trifolii.

| Antigens. | Sera. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 36 | 108 | 64 | 94 | 61 | 161 | 111 | 160 | 91 | 46 | 204 | 157 |
| 36 | *4* [4] | 4 [4] | 2 | – | –⁻ | – | –⁻ | – | –⁻ | –⁻ | – | – |
| 108 | 4 [4] | *4* [4] | 4 | – – – | –⁻ | – | –⁻ – | – | –⁻ | –⁻ | – | – |
| 64 | 3 [4] | 4 | *4* | – – | –⁻ | – | – | – | – | –⁻ | – | – |
| 94 | –⁻ | – | – | *3* | 3 [2] | 3 | 2 | 3 | – | –⁻ | – | – |
| 61 | –⁻ | –⁻ | – | 3 | *4* [3] | 3 | 2 [3] | – | – | – | – | – |
| 161 | –⁻ | – | – | 3 | 2⁻ | *4* | – | 3 | – | –⁻ | – | – |
| 111 | –⁻ | –⁻ | – | 2 | 4 [3] | – | *4* [3] | – | –⁻ | –⁻ | – | – |
| 160 | –⁻ | – | – | 3 | –⁻ | 2 | – | *4* | – | –⁻ | – | – |
| 91 | –⁻ | –⁻ | – | – | –⁻ | – | –⁻ | – | *4* [4] | 4 [3] | – | – |
| 46 | –⁻ | – | – | – | –⁻ | – | –⁻ | – | 4 [4] | *4* [4] | – | – |
| 204 | –⁻ | – | – | – | –⁻ | – | – | – | – | –⁻ | *4* | – |
| 157 | –⁻ | – | – | – | –⁻ | – | – | – | – | –⁻ | – | *4* |

*Note.*—Numbers indicate highest dilutions showing agglutination, viz. 1 = 1/25 to 1/50, 2 = 1/100 to 1/200, 3 = 1/400 to 1/800, 4 = 1/1600 to 1/3200 or greater.
Top right-hand figure shows earlier result.

TABLE 3.
*Flagellar Cross Reactions of* Rhizobium meliloti.

| Antigens. | Sera. | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 8 | 12 | 27 62 | 51 | 52 | 66 | 74 | 76 | 102 | 126 | 134 | 10 | 47 | 101 |
| 7 | *4* | 4 | 4 | 4 [4] | 4 | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 2 | – [2] | – |
| 8 | 4 | *4* | 4 | 4 [4] | 4 | 4 | 4 | 4 | 2 | 4 | 4 | 4 | 1 | 1 [1] | – |
| 12 | 4 | 4 | *4* | 4 [4] | 4 | 4 | 4 | 4 | 3 | 4 | 4 | 4 | 1 | – [1] | – |
| 27, 62 | 4 | 4 | 4 | *4* [4] | 4 | 4 | 4 [4] | 4 [4] | 3 | 4 [4] | 4 | 4 | 1 | – [1] | – |
| 51 | 4 | 4 | 4 | 4 [4] | *4* | 4 | 4 | 4 | 2 | 4 | 3 | 4 | 2 | 1 [1] | – |
| 52 | 4 | 4 | 4 | 4 | 4 | *4* | 4 | 4 | 3 | 4 | 4 | 4 | 1 | – | – |
| 66 | 4 | 4 | 4 | 4 [4] | 4 | 4 | *4* [4] | 4 [4] | 3 | 4 [4] | 4 | 4 | 2 | 2 – | – |
| 74 | 4 | 4 | 4 | 4 [4] | 4 | 4 | 4 [4] | *4* [4] | 3 | 4 [4] | 4 | 4 | 1 | 1 [1] | – |
| 76 | 4 | 4 | 4 | 4 [4] | 3 | 4 | 4 | 4 | *3* | 4 | 4 | 4 | 1 | 1 [1] | – |
| 102 | 4 | 4 | 4 | 4 [4] | 4 | 4 | 4 [4] | 4 [4] | 3 | *4* [4] | 4 | 4 | 1 | 1 [1] | – |
| 126 | 4 | 4 | 4 | 4 [4] | 4 | 4 | 4 | 4 | 4 | 4 | *4* | 4 | 1 | – – | – |
| 134 | 4 | 4 | 4 | 4 [4] | 4 | 4 | 4 | 4 | 3 | 4 | 4 | *4* | 2 | – [1] | – |
| 10 | 2 | 1 | 2 | ± [2] | – | 1 | 1 | ± | – | 2 | 1 | 2 | *4* | 3 [4] | – |
| 47 | 2 | 2 | 3 | 1 [1] | 3 | 3 | 1 [2] | 1 [1] | – | 1 [2] | 3 | 3 | 4 | *3* [3] | – |
| 101 | – | – | – | – – | – | – | – | – | – | – | – | – | – | – – | *5* |

*Note.*—± = variable result at lowest titre; code otherwise as for Table 1.

*Strains of Rh. meliloti.*—Tables 3 and 4 give flagellar and somatic reactions of sixteen strains.

Most of the flagellar reactions are those previously seen in strain 27, but strain 10 has the major antigen of 47, and 101 is different from both these groups. Using the notation of the first paper, 10 and 47 are described as Ab, the 27 group as bC and 101 is now characterized as D. The symbol *b* is used to describe a minor antigen that is commonly shared by members of the first two groups.

Somatic antigens provide a basis for further division. Approximate groups are represented by:

7, 27 and 62, 47
52, 102
126, 8, 12
134, 74
66
10, 76, 101
51

although there is a certain amount of cross reaction beyond these limits. Cross absorption tests have shown 7, 27 and 62 to be identical; they have, however, an antigen additional to those of 47 and the latter has an antigen not common to them. No. 76 also appears to have the same antigenic constitution as 101, otherwise there are differences even within the members of the groups set out. The group represented by 126, 8 and 12 shows a wide range of reactivity when they constitute the antigen, but outside the group itself reciprocal tests are mostly negative. It seems that the cells of

TABLE 4.
*Somatic Cross Reactions of* Rhizobium meliloti.

| Antigens. | Sera. | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 | 27 62 | 47 | 52 | 102 | 126 | 8 | 12 | 134 | 74 | 66 | 10 | 76 | 101 | 51 |
| 7 | *4* | $4^{4}$ | $1^{2}$ | – | $-^{-}$ | – | – | – | – | $-^{-}$ | $-^{-}$ | – | – | – | – |
| 27, 62 | 3 | $4^{4}$ | $2^{2}$ | – | $-^{-}$ | – | – | – | – | $-^{-}$ | $-^{-}$ | – | – | – | – |
| 47 | 3 | $3^{3}$ | $3^{4}$ | 1 | $\pm^{1}$ | – | – | – | ± | $-^{-}$ | $-^{-}$ | – | – | – | – |
| 52 | 2 | – | 1 | *4* | 2 | 4 | ± | – | ± | – | – | – | – | – | – |
| 102 | – | $-^{-}$ | $1^{1}$ | 3 | $4^{4}$ | 1 | – | – | – | $-^{-}$ | $-^{-}$ | – | – | – | – |
| 126 | 1 | $\pm^{-}$ | $2^{-}$ | 2 | $2^{3}$ | *4* | 3 | 3 | 2 | $2^{-}$ | $2^{-}$ | 3 | 2 | – | 3 |
| 8 | 1 | $-^{2}$ | $3^{1}$ | 2 | $2^{2}$ | 4 | *4* | 4 | 3 | $2^{2}$ | $2^{3}$ | 2 | 2 | 3 | 3 |
| 12 | – | $-^{2}$ | $4^{3}$ | 2 | $1^{2}$ | 4 | 4 | *5* | 4 | $1^{2}$ | $2^{3}$ | 4 | 2 | 3 | 3 |
| 134 | – | $-^{-}$ | $-^{-}$ | – | $-^{-}$ | 1 | – | – | *4* | $2^{3}$ | $-\pm$ | – | – | – | – |
| 74 | – | $-^{-}$ | $-^{-}$ | – | $-^{-}$ | – | – | – | 3 | $2^{3}$ | $1^{1}$ | – | – | – | – |
| 66 | – | $-^{-}$ | $-^{-}$ | – | $-^{-}$ | – | – | – | 1 | $-^{-}$ | $2^{4}$ | – | – | – | – |
| 10 | – | $-^{-}$ | $\pm\pm$ | 1 | $-^{-}$ | ± | – | ± | ± | $-^{-}$ | $-^{-}$ | *4* | 3 | 4 | 1 |
| 76 | – | $-^{-}$ | $-^{-}$ | – | $-^{-}$ | – | – | – | – | $-^{-}$ | $-^{-}$ | 2 | *3* | 3 | – |
| 101 | – | $-^{-}$ | $-^{-}$ | – | $-^{-}$ | – | – | – | – | $-^{-}$ | $-^{-}$ | 3 | 3 | *3* | – |
| 51 | – | $-^{-}$ | $-^{-}$ | – | $-^{-}$ | – | – | – | – | $-^{-}$ | $-^{-}$ | 1 | ± | – | *2* |

*Note.*—± = variable result at lowest titre, code otherwise as for Table 1.

this group are relatively easy to agglutinate, perhaps by a less specific antibody, and only reactions that have reciprocal tests in agreement have been used in stating somatic antigens. Cases 52 against serum 7, 66 *vs.* 134, 10 *vs.* 52, in which the reciprocal test is negative, have also been ignored. Antigen II, previously postulated as common to 66 and 74, would, on similar grounds, be regarded as a doubtful or minor antigen.

The data for cross reaction and absorption tests can be symbolized thus:

| Isolate. | Minimal Antigenic Constitution. Flagellar. | Somatic. |
|---|---|---|
| 47 | Ab | I, III |
| 10 | Ab | X, XI |
| 7, 27, 62 | bC | I, IV |
| 52 | bC | III, V, XII |
| 102 | bC | III, V, XIII |
| 126 | bC | V, VIII, IX |
| 8 | bC | VIII, XIV |
| 12 | bC | VIII, XV |
| 134 | bC | VI, IX |
| 74 | bC | II, VI |
| 66 | bC | II, VII |
| 76 | bC | X |
| 51 | bC | XI |
| 101 | D | X |

Sera of the strains underlined have been used in typing field isolates (Purchase, Vincent and Ward, 1950). These include the three flagellar groupings and all the somatic antigens so far revealed.

## Discussion.

These results emphasize the serological heterogeneity that exists within a "species" of *Rhizobium*. There are notably fewer differences in flagellar reaction than somatic, and distinction between the two types is obviously desirable for the more specific recognition of a strain. It is interesting to observe how, with both clover and medic isolates, the one somatic antigen can occur with any of the flagellar groupings.

*Acknowledgements.*

The work reported in this paper has been supported in part by grants from the Commonwealth Research Committee and the Rural Bank of New South Wales.

*References.*

Hughes, D. Q., and Vincent, J. M., 1942.—Serological Studies of the root-nodule bacteria. III. Tests of neighbouring strains of the same species. Proc. Linn. Soc. N.S.W., 67: 142-152.

Kleczkowski, A., and Thornton, H. G., 1944.—A serological study of root-nodule bacteria from pea and clover inoculation groups. *J. Bact.*, 48: 661-672.

Nutman, P. S., 1946.—Variation within strains of clover nodule bacteria in the size of nodules produced and in the effectivity of the symbiosis. *J. Bact.*, 51: 411-432.

Purchase, Hilary F., and Vincent, J. M., 1949.—A detailed study of the field distribution of strains of clover nodule bacteria. Proc. Linn. Soc. N.S.W., 74: 227-236.

Purchase, Hilary F., Vincent, J. M., and Ward, Lawrie M., 1950.—The field distribution of strains of nodule bacteria from species of *Medicago*. *Aust. J. Agr. Res.*, 1951 (in press).

Vincent, J. M., 1941.—Serological studies of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. Proc. Linn. Soc. N.S.W., 66: 145-154.

————, 1942.—Serological studies of the root-nodule bacteria. II. Strains of *Rhizobium trifolii*. *Idem.*, 67: 82-86.

————, 1944.—Variation in the nitrogen fixing property of *Rhizobium trifolii*. *Nature*, 153: 496-497.

————, 1945.—Host specificity amongst root-nodule bacteria isolated from several clover species. *J. Aust. Inst. Agric. Sci.*, 11: 121-127.